Asian Journal of Andrology (2013) 15, 328–332

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RESEARCH HIGHLIGHT

Neuroendocrine differentiation of prostate cancer

Zhen Li, Clark J Chen, Jason K Wang, Elaine Hsia, Wei Li, Jill Squires, Yin Sun and Jiaoti Huang

Asian Journal of Andrology (2013) **15,** 328–332; doi:10.1038/aja.2013.7; published online 18 March 2013

Benign prostate and prostatic adenocarcinoma contain rare quiescent neuroendocrine cells while small cell neuroendocrine carcinoma (SCNC), a variant form of prostate cancer, contains highly proliferative neuroendocrine tumor cells. We provide evidence that IL-8-CXCR2-P53 pathway keeps the NE cells in a quiescent state normally. P53 mutation inactivates this pathway, resulting in hyper-proliferation and aggressive behavior of the NE cells in SCNC. Therefore, we have identified potential cells of origin and a molecular target for prostatic SCNC that are different from those of adenocarcinoma, which explains SCNC's distinct biology and the clinical observation that it does not respond to hormonal therapy.

Prostate cancer is a major health risk for men in Western countries as it is the most common malignancy and the second leading cause of cancer-related deaths. In some countries where prostate cancer is traditionally uncommon such as China, the incidence of prostate cancer has also shown a dramatic increase in recent years.

Low grade, organ-confined prostate cancers are curable by surgery or radiation therapy. For patients with recurrent or metastatic prostate cancer, hormonal therapy, by inhibiting androgen production and/or blocking androgen receptor (AR) function, is the treatment of choice. Unfortunately, hormonal therapy is not curative and the cancer nearly always recurs after an initial period of response and inevitably progresses to the castration resistant stage. Newer agents have been developed to inhibit intratumoral androgen production or more effectively block AR function, but resistance occurs quickly. In this article, we will focus our discussion on cellular heterogeneity of human prostate cancer and molecular mechanisms responsible for the development of small cell neuroendocrine carcinoma (SCNC), a lethal form of prostate cancer often seen in patients who have received hormonal therapy.

Departments of Pathology and Urology, Jonsson Comprehensive Cancer Center and Broad Center for Regenerative Medicine and Stem Cell Research, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095–1732, USA
Correspondence: Dr J Huang
(JiaotiHuang@mednet.ucla.edu)
Received: 30 November 2012; Revised: 8 January 2013; Accepted: 9 January 2013; Published online: 18 March 2013

NEUROENDOCRINE CELLS IN BENIGN PROSTATE

Prostate is a glandular organ in which the epithelial cells form glands or ducts. Under the light microscope, two types of epithelial cells are easily identifiable in the prostate: (i) secretory cells (or luminal cells) that produce secreted proteins including prostate-specific antigen (PSA); and (ii) basal cells that likely function as reserve cells. There is a third, minor epithelial cell type called neuroendocrine (NE) cells that are scattered among the basal and luminal cells (**Figure 1**, left panel) and constitute ~1% of the total epithelial cell population.[1–3] The NE cells can be identified by electron microscopy showing distinct ultrastructural morphology and intracytoplasmic dense-core secretory granules. More commonly, they are detected by immunohistochemistry (IHC) with antibodies against NE markers such as Chromogranin A or Synaptophysin with the former being more sensitive and specific.[1–3] In mice, NE cells are concentrated around the proximal urethra while not commonly seen in the various lobes of the prostate. Since NE cells are present in every single benign human prostate, they are presumed to play important roles in prostate development or function. However, little experimental evidence exists that provides significant insights into their function.

NEUROENDOCRINE CELLS IN PROSTATIC ADENOCARCINOMA

Prostatic adenocarcinoma is the most common malignant tumor type in human prostate, accounting for well over 90% of all prostate cancers. Histologically, it is characterized by uncontrolled proliferation of malignant secretory-type tumor cells and a lack of basal cells. Interestingly, every single case of prostatic adenocarcinoma also contains scattered NE tumor cells (**Figure 1**, middle panel) and the number of NE cells varies from case to case. On average, NE cells account for no more than 1% of all tumor cells, although rare cases contain abundant NE cells. It remains controversial whether the number of NE tumor cells correlates with tumor grade and stage. The main difficulty in resolving this issue is that IHC staining is required to highlight the unevenly distributed NE cells and it is impractical to stain the entire tumor to accurately count all NE cells. On the other hand, it is widely accepted that hormonal therapy for adenocarcinoma leads to increased NE tumor cells, and abundant NE tumor cells are seen in most castration resistant prostate cancers (**Figure 1**, right panel). The mechanism for the increase in the NE cell component of adenocarcinoma after hormonal therapy is unclear. Unlike the bulk, secretory type cancer cells, NE tumor cells do not express AR[4] and are independent of androgen. As a result, while the bulk tumor cells undergo apoptosis after androgen ablation, the NE tumor cells continue to survive and their relative percentage in the tumor increases (**Figure 2**). There has also been evidence that the normally quiescent NE tumor cells may proliferate under androgen-deprived conditions,[5] so it is possible that there is also an expansion of this cell type resulting in an increase in the absolute number of NE tumor cells.

The consequence of increased NE tumor cells after hormonal therapy is of significant research interest. Experimental evidence has been presented showing that NE tumor cells can promote androgen-independent growth and tumorigenesis,[6] and invasion and metastasis of prostate cancer cells,[7] likely through

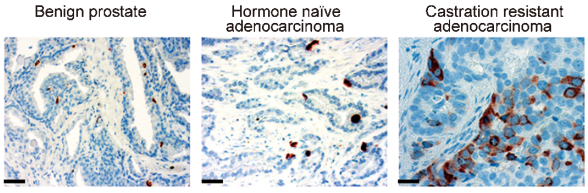

Figure 1 Neuroendcorine cells in benign prostate and prostatic adenocarcinoma. Left panel shows scattered neuroendocrine cells (brown staining) in benign prostate as highlighted by IHC staining with an anti-chromogranin A antibody. Middle panel shows scattered neuroendocrine cells in a case of prostatic adenocarcinoma that is hormone naive. Right panel shows abundant neuroendocrine cells in a case of castration resistant adenocarcinoma. IHC, immunohistochemistry. Scale bar=20 μm.

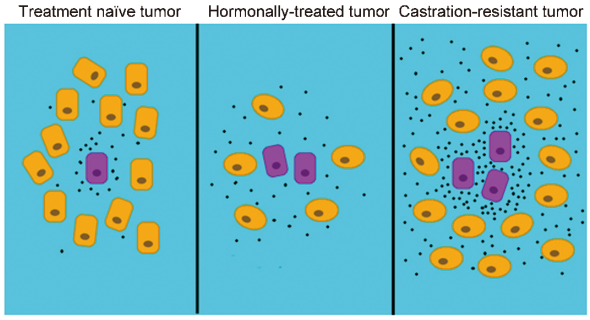

Figure 2 A model of the function of cellular heterogeneity in prostate cancer. The left panel shows a treatment naive tumor in which the majority of the tumor cells are luminal type tumor cells (yellow) with few neuroendocrine cells (pink). The middle panel shows that hormonal therapy induces apoptosis of the luminal type tumor cells and an increase in the neuroendocrine cells. The right panel shows that in the castration resistant stage, there is a marked increase in neuroendocrine cells which establish a paracrine network and stimulate proliferation of the luminal type cancer cells in an androgen-deprived environment.

their secreted products.[8,9] Studies over the years have shown that NE cells secret biogenic amines, neuropeptides and cytokines, while the bulk, non-NE tumor cells express receptors for many of the NE cell products.[1–3] Therefore, it has been hypothesized that hormonal therapy for adenocarcinoma leads to increased NE tumor cells, which establish paracrine networks within the tumor and stimulate proliferation, invasion and metastasis of the bulk, non-NE tumor cells in the androgen-deprived environment, contributing to the progression of the tumor to the castration resistant stage (**Figure 2**). Hence, NE tumor cells may be important therapeutic targets.

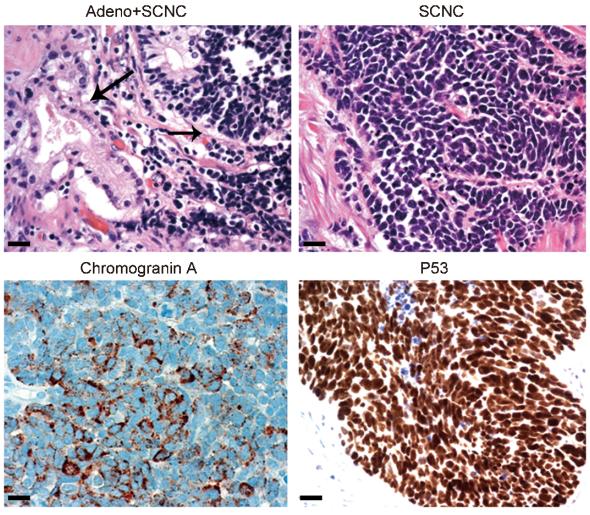

Figure 3 SCNC of the prostate. Upper left panel shows a case of mixed tumor with components of adenocarcinoma (long arrow) and SCNC. Upper right panel shows a high power view of SCNC with tumor cells demonstrating high-grade neuroendocrine features. Lower left panel shows IHC staining of SCNC with an anti-chromogranin A antibody. Lower right panel shows IHC staining with an anti-P52 antibody. IHC, immunohistochemistry; SCNC, small cell neuroendocrine carcinoma. Scale bar=20 μm.

IL-8 SIGNALING IN NEUROENDOCRINE CELLS OF PROSTATIC ADENOCARCINOMA

LNCaP is a classic prostate cancer cell line that possesses the important characteristics of secretory type adenocarcinoma cells. They express AR and secret PSA and their proliferation is dependent on continued presence of androgen. Withdrawal of androgen in the culture media, usually by replacing normal fetal bovine serum with charcoal-stripped fetal bovine serum, leads to growth arrest and eventual acquisition of NE phenotype in LNCaP cells,[10] including elongation of cell bodies mimicking neuronal cells and expression of NE markers such as neuron-specific enolase. Dr Kung's group at UC Davis showed that adding IL-8 to androgen-deprived media promoted proliferation and migration of LNCaP cells, suggesting that IL-8 may be one of the factors contributing to the progression of adenocarcinoma to the castration resistant state.[11] We were intrigued by this finding and studied the source of IL-8 in human prostatic adenocarcinoma. We stained adenocarcinoma tissue with an antibody against IL-8 and interestingly, only scattered tumor cells stained strongly, while the vast majority of the tumor cells were entirely negative. By staining serial sections of adenocarcinoma tissue with anti-IL-8 and anti-Chromogranin A antibodies, respectively, we confirmed that NE tumors cells are the only tumor cells that produce IL-8. There are two known receptors for IL-8 in human tissues, CXCR1 and CXCR2. While benign secretory type epithelial cells of the prostate do not express CXCR1, this receptor is over-expressed in adenocarcinoma cells. It is therefore reasonable to propose that a paracrine activation of CXCR1 (expressed by bulk tumor cells) by IL-8 (secreted by NE tumor cells) may contribute to androgen-independent proliferation of prostate cancer in patients treated with hormonal therapy.

Another receptor for IL-8 is CXCR2 which shares significant homology with CXCR1. We also immunohistochemically stained adenocarcinoma tissue for the expression of CXCR2 and our results demonstrated that similar to IL-8, CXCR2 was exclusively expressed in the NE tumor cells of adenocarcinoma. Since NE cells in adenocarcinoma express IL-8 and its receptor CXCR2, we hypothesized that there is an autocrine

interaction which may be critical for certain aspects of the NE cell biology.[12]

In addition to being negative for AR and PSA, an important feature of NE cells in adenocarcinoma is that they are normally quiescent,[4] which is unique among the cancer cells. The molecular mechanism that maintains the NE tumor cells in a quiescent state was unknown. A few years after our finding that NE cells express IL-8 and CXCR2, another group reported that fibroblasts that undergo senescence overexpress IL-8 and CXCR2 and the autocrine action is critical for the induction of senescence. The authors also showed that the induction of cellular senescence by IL-8-CXCR2 signaling is mediated by P53.[13] We reasoned that a similar mechanism may also exist in NE cells, that is, IL-8-CXCR2 signaling may keep NE cells of benign prostate and adenocarcinoma in a quiescent state. We further hypothesized that P53 may similarly mediate IL-8/CXCR2 signaling and the IL-8/CXCR2/P53 signaling pathway may represent a major growth inhibitory pathway of the NE cells.

CELL OF ORIGIN AND MOLECULAR BASIS OF PROSTATIC SMALL CELL NEUROENDOCRINE CARCINOMA

While the above hypothesis is interesting in terms of understanding the molecular physiology of the NE cells, it has important clinical implications as well. Clinically, a small percentage of prostate cancers is classified as SCNC in which the tumor cells are all NE cells (**Figure 3**). In contrast to the NE cells in benign prostate and adenocarcinoma that are normally quiescent, NE tumor cells in SCNC are highly proliferative and aggressive, often leading to widespread metastasis and death within months of the initial diagnosis. SCNC can arise *de novo* in men without a history of adenocarcinoma but more commonly, it occurs as a recurrent tumor in men with a history of adenocarcinoma and treated with hormonal therapy. SCNC can occur in a pure form, but it is often found in a mixed tumor where it coexists with conventional adenocarcinoma (**Figure 3**, upper left). In contrast to prostatic adenocarcinoma which forms glands, SCNC has a solid, sheet-like growth pattern but no glandular formation. Tumor cells are small, with fine chromatin pattern, scant cytoplasm and nuclear molding. Mitotic figures, crush artifact and tumor necrosis are frequent findings[14–16] (**Figure 3**, upper right).

The reported incidence of prostatic SCNC is low (~1% of all prostate cancers), but this disease is likely significantly underdiagnosed.[17] A main reason is that patients with known prostatic adenocarcinoma whose diseases have become advanced and widely metastatic on imaging studies usually do not undergo tissue diagnosis. Another reason is that SCNC is morphologically very different from prostatic adenocarcinoma and often metastasizes to visceral organs, while adenocarcinoma typically involves bone and lymph nodes. As a result, a connection between metastatic SCNC and the original prostatic adenocarcinoma may not always be established. On the other hand, accurate diagnosis of SCNC is important as such tumors do not respond to hormonal therapy targeting the AR signaling pathway[18] which produces a therapeutic response, at least initially, in nearly all cases of prostatic adenocarcinoma.[19]

The molecular mechanism for prostatic SCNC was unclear, although the phenomenon of prostatic adenocarcinoma recurring as SCNC had been well recognized clinically. Because IL-8/CXCR2/P53 pathway may keep the NE cells in a quiescent state, we hypothesized that inactivation of this pathway may remove a major growth inhibitory mechanism, leading to rapid proliferation and aggressive behavior of the malignant NE cells and resulting in the development of SCNC.[20] This hypothesis has two important components: (i) The cell of origin of prostatic SCNC may be the NE cells of either benign prostate or prostatic adenocarcinoma, with the latter more likely; and (ii) P53 mutation may be a critical molecular event for the development of SCNC.

To test this hypothesis, we developed stable clones of LNCaP cells that express high levels of CXCR2 (LNCaP/CXCR2 cells). We examined transcriptional response of LNCaP/CXCR2 cells to IL-8 stimulation and found that numerous genes were up- or downregulated after activation of CXCR2. Functional gene family analysis showed that among the genes whose expression increased significantly in response to IL-8 stimulation were those controlling the G_1/S and G_2/M transition check points, while many genes downregulated after IL-8 stimulation are involved in cell morphology and development. Importantly, the expression of many genes involved in P53 signaling was significantly altered after IL-8 stimulation.[20]

In support of our hypothesis, IL-8 inhibited the proliferation of LNCaP/CXCR2 cells but not the parental LNCaP cells. In addition, P53 was required for the growth-inhibitory function of CXCR2 signaling, as knockdown of P53 by siRNA abolished the growth inhibition of LNCaP/CXCR2 cells by IL-8.[20]

The above observations were confirmed with another commonly used prostate cancer cell line PC3. Our group has recently shown that PC3 cells possess features of prostatic SCNC in that these cells are negative for luminal differentiation markers AR and PSA but positive for NE markers.[21] Similar to NE cells in benign prostate and prostatic adenocarcinoma, PC3 cells express IL-8[22] and CXCR2.[23] Contrary to NE cells in benign prostate and prostatic adenocarcinoma, PC3 cells are highly proliferative and aggressive which is similar to NE tumor cells of human prostatic SCNC. We hypothesized that the loss of the growth inhibitory function of the IL-8/CXCR2/p53 pathway may be responsible for the rapid proliferation and aggressive behavior of PC3 cells. Consistent with our hypothesis, it has been reported that PC3 cells, unlike LNCaP cells, contain a p53 mutation.[24] When we re-introduced wild-type P53 into PC3 cells, IL-8 treatment inhibited growth of PC3 cells.[20]

To provide further evidence that P53 mutation is a critical molecular event in the pathogenesis of prostatic SCNC, we performed IHC staining of benign human prostate, prostatic adenocarcinoma and prostatic SCNC. Positive nuclear staining for P53 was used as a surrogate marker for P53 mutation since it is well known that mutant P53 has prolonged half-life and the P53 protein becomes detectable in the nucleus by IHC staining. We used a tissue microarray containing adenocarcinoma and benign prostate tissue from 150 cases and adjacent sections were stained with antibodies against Chromogranin A and P53, respectively. In both types of tissue, the scattered NE cells and the scattered P53-positive cells did not overlap, suggesting that in benign prostate and prostatic adenocarcinoma, the NE cells likely have wild-type p53. In contrast, when regular sections from 31 cases of prostatic SCNC were stained for P53, strong and diffuse nuclear positivity was observed in the majority of the cases, strongly suggesting frequent P53 mutation in such tumors. We obtained formalin-fixed, paraffin-embedded tissue from seven cases of prostatic SCNC and extracted genomic DNA from them. Sequencing of exons 5–10 showed that five out of seven tumor samples contained an identical allele of a missense transition converting G to A at position 747, changing the negatively charged aspartic acid to hydrophilic amino-acid asparagine at amino acid 184, supporting our hypothesis that P53 mutation is likely a critical event in the pathogenesis of prostatic SCNC.

The results of the above studies reveal the likely cell of origin and the molecular basis of prostatic SCNC. NE cells may play important roles in benign prostate and treatment naive adenocarcinoma, although little detail is known. Because these cells are AR negative and androgen-independent, they are resistant to hormonal therapy which induces apoptosis of the bulk, non-NE tumor cells. Besides causing a relative increase in the proportion of NE cells in the tumor, hormonal therapy may also induce proliferation of the normally quiescent NE cells,[5] resulting in an increase in the absolute number of NE cells in the tumor. We propose that during the process, a P53 mutation occurs in the NE cells, inactivating the growth-inhibitory IL-8/CXCR2/P53 pathway, resulting in rapid proliferation and aggressive behavior of the NE cells and the development of SCNC[20] (**Figure 4**). In fact, IL-8/CXCR2 signaling may become growth-stimulating in the absence of functional P53.[20] In our opinion, SCNC is an underdiagnosed entity for reasons discussed above. With the advent of new drugs that represent super-AR blockers (e.g., MDV3100) or inhibitors of intratumoral androgen synthesis (e.g., abiraterone), we only expect an even higher incidence of prostatic SCNC. Because the NE tumor cells do not express AR and are independent of androgen, SCNC does not respond to therapies targeting AR pathway. Therefore, it is considered a disease that is generally lethal without effective treatment options.[25]

The above hypothesis, although interesting and potentially very important, still presents an incomplete picture of SCNC For example, it remains unclear if SCNC only arises from NE tumor cells of adenocarcinoma, or the NE cells in benign prostate can also be cells of origin for SCNC. Most commonly, prostatic SCNC occurs in patients with a history of adenocarcinoma treated with hormonal therapy, suggesting NE tumor cells of adenocarcinoma as the likely cell of origin. This notion is supported by pathological observation that prostatic SCNC often coexists with a conventional prostatic adenocarcinoma (**Figure 3**, upper left) and pure SCNC is very rare. However, clinically, some patients without a history of adenocarcinoma can present with prostatic SCNC, suggesting that NE cells in benign prostate can also give rise to SCNC. The caveat is that these patients may have had a pre-existing undiagnosed adenocarcinoma which has been completely replaced by the rapidly proliferating tumor cells of SCNC.

ANIMAL MODELS OF PROSTATE SCNC AND THE UNCERTAIN ROLE OF *RB* GENE

Our observations provide insights into the mechanism of pathogenesis of the well-known transgenic adenocarcinoma of mouse prostate (TRAMP) model. This model was established by overexpression of SV40 early genes under the control of the AR-responsive *probasin* promoter.[26] It had been known for a long time that TRAMP tumors resemble human prostatic SCNC morphologically, but a satisfactory explanation for the unusual histology had not been provided. We believe that although AR is considered a marker of prostate luminal differentiation, it is possible that a low level of AR is present in NE cells at some point of mouse prostate development and is sufficient to activate the *probasin* promoter. We hypothesize that in these animals, inactivation of P53 in NE cells of the prostate leads to malignant transformation and rapid proliferation of NE cells, resulting in the development of a malignant tumor with abundant NE tumor cells mimicking human prostatic SCNC.

SV40 T antigen inactivates both P53 and Rb in TRAMP tumors. Nikitin's group[27] has developed a $p53^{-/-}Rb^{-/-}$ double knockout mouse model in which *probasin* promoter was used to drive the expression of the Cre recombinase, and the resulting tumors show similar SCNC morphology. Their study showed that both P53 and Rb need to be inactivated in order for invasive tumors to develop. Therefore, it remains unclear if inactivation of Rb as well as p53 in NE cells is required for the development of prostatic SCNC in human patients.

Notably, in both TRAMP and $p53^{-/-}Rb^{-/-}$ double knockout models, there are also areas of glandular formation resembling adenocarcinoma, which probably results from inactivation of p53 and/or Rb in the luminal epithelial cells. In most of the TRAMP tumors we have examined, NE tumor cells predominate, likely reflecting the highly proliferative nature of the malignant NE tumor cells which can easily obliterate the adenocarcinoma areas.

Another important issue is the nature of p53 mutation. We observed the same mutation in five of seven SCNC samples sequenced, but this is unlikely the only mutation for all such tumors. de Marzo's group[28] reported positive nuclear staining for p53 and a p53 mutation in a case of SCNC intermixed with adenocarcinoma. The p53 mutation discovered in their study differs from the one identified in our cases, suggesting that a variety of p53 mutations may be found if a large number of SCNCs are sequenced.

Prostatic SCNC has gained increased recognition as an important disease entity and two recent studies have used powerful sequencing technologies for such tumors. Rubin's group[29] discovered that a large number of prostatic SCNCs have significant overexpression and gene amplification of Aurora kinase A and MYCN and the former may serve as a potentially useful therapeutic target Collins's group[30] found REST transcriptional complex to be critical in the development of prostatic SCNC Such diverse findings have undoubtedly increased our understanding of this important disease and collectively may eventually help to find novel therapies.

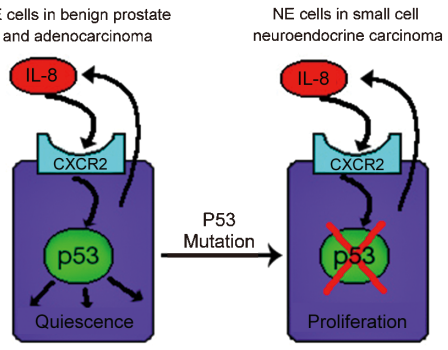

Figure 4 A model of the molecular pathway regulating the proliferation of neuroendocrine cells. The left panel shows a neuroendocrine cell in benign prostate and prostatic adenocarcinoma. Autocrine stimulation of CXCR2 by IL-8 activates P53 and inhibits cell proliferation. On the right is a NE tumor cell in prostatic small cell carcinoma. A p53 mutation inactivates the IL-8/CXCR2/p53 pathway and removes a major growth inhibitory signal, leading to rapid proliferation of the NE cell.

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